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Ultrasound enhances lipase-catalyzed synthesis of poly (ethylene glutarate)

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ABSTRACT

The present work explores the best conditions for the enzymatic synthesis of poly (ethylene glutarate) for the first time. The start-up materials are the liquids; diethyl glutarate and ethylene glycol diacetate, without the need of addition of extra solvent. The reactions are catalyzed by lipase B from *Candida antarctica* immobilized on glycidyl methacrylate-ter-divinylbenzene-ter-ethylene glycol dimethacrylate at 40 °C during 18 h in water bath with mechanical stirring or 1 h in ultrasonic bath followed by 6 h in vacuum in both the cases for evaporation of ethyl acetate. The application of ultrasound significantly intensified the polyesterification reaction with reduction of the processing time from 24 h to 7 h. The same degree of polymerization was obtained for the same enzyme loading in less time of reaction when using the ultrasound treatment. The degree of polymerization for long-term polyesterification was improved approximately 8-fold due to the presence of sonication during the reaction. The highest degree of polymerization achieved was 31, with a monomer conversion of 96.77%. The ultrasound treatment demonstrated to be an effective green approach to intensify the polyesterification reaction with enhanced initial kinetics and high degree of polymerization.

1. Introduction

Enzyme-based polymerization reactions provide greener alternative to the conventional chemical processes [1]. Enzymatic reactions minimize the formation of undesirable by-products; avoid harsh conditions and the requirement of metallic catalysts [2,3]. Enzymes can be used to produce specialty functional polymers [4]. When immobilized on firm, solid and porous supports enzymes can have better activity and stability [5,6]. Lipases are described as bio-catalysts in solvent [7–10] and in emulsion systems [9,11,12]. Potentially a solvent-free enzymatic system can offer better processing conditions without further complex purification process [10,11,13,14]. The use of solid immobilized enzymes in solvent free system where the reactants are the solvents itself might yield slow reaction rates due to the limiting diffusion between reactants and mass transfer limitations. The use of ultrasound might overcome these disadvantages [12,15]. The observed intensification is mainly due to the cavitational effects, such as increased local temperature and pressure as well as the generation of micro level mixing and turbulence conditions [16,17]. The ultrasonic irradiation produces alternate compression and rarefaction cycles at frequency of 20,000 cycles/s. That fortifies the interaction between phases by cavitation and/or formation of micro flows in liquids, which is initiated by violent collapse of small bubbles or voids in liquids as a result of pressure fluctuation. Additionally, ultrasound also improves mixing, shearing, and mass transfer in the reaction solution or suspensions [14–20]. Moreover cavitation may enhance polymerization by temporarily dispersing aggregates and preventing the formation of layers of compounds surrounding the immobilized enzyme due the micro scale turbulence generated by the cavitational effects [17,18,21–24]. In the case of polymerization, the heterogeneity in the system due to the use of different

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phases results in significant mass transfer resistances which can be eliminated by the use of ultrasonic irradiations.

The use of immobilized lipases as catalysts in ultrasound-assisted esterification reactions of short carboxylic acids to obtain esters has been reported by Rodrigues et al. [19]. Under optimized conditions there was an increase on the conversion of the reagents using ultrasounds. Despite the improvement of the esterification reaction using immobilized enzyme and ultrasounds the processes was not solvent free with a single ester as final product.

Although ultrasound-assisted lipase-catalyzed synthesis of polyester is an innovative approach, very few works have been reported in the literature showing better efficacy. For instance, studies related to the ultrasound assisted lipase-catalyzed synthesis of poly-6-hydroxyhexanoate revealed that sonication improved the monomer conversion by 63% and resulted in a polymer product with a narrower molecular weight distribution and a higher degree of crystallinity as compared to the conventional non-sonicated route [19]. In another study of enzymatic synthesis of poly-ε-caprolactone, it was reported only slightly of ultrasound enhancement of the rate constant for chain propagation (K_p) of the poly-ε-caprolactone. The acoustic effects also allowed the reaction to continue longer based on the control over the viscosity as compared to the non-sonicated process where it became impossible to operate due to the highly elevated reaction mixture viscosity (>2000 times increase from initial viscosity) [25].

In the present study, we investigated for the first time the synthesis of aliphatic polyester from diethyl glutarate and ethylene glycol diacetate using immobilized lipase B from *Candida antarctica* in a solvent free reaction. The effect of ultrasound on the initial reaction kinetics (concentrations analyzed by gas chromatography-flame ionization detector (GC-FID) was established in terms of the comparison with the conventional approach of using a shaker with controlled temperature. The effect of reaction operating parameters on the time of reaction, degree of polymerization and monomer conversion rate were analyzed using the measurements based on the use of nuclear magnetic resonance (NMR).

2. Materials and methods

2.1. Materials

Fermase CALB™ 10,000, a commercial *C. antarctica* lipase B (CALB) immobilized on glycidyl methacrylate-ter-divinylbenzene-ter-ethylene glycol dimethacrylate (particle size of 150–300 μm, pore volume of 1.32 cm^3/g, bulk density of 0.54 g/cm^3 and an activity of 8000 propyl laurate units) was obtained as a gift sample from Fermenta Biotech Ltd., Mumbai, India. Diethyl glutarate (DG) (purity ⩾99%), ethylene glycol diacetate (EGD) (purity 99%), chloroform (AR), ethyl acetate (HPLC, 99.9%), petroleum ether (AR, 40–60 °C), tetrahydrofuran (AR) were obtained from Sigma–Aldrich, Co., Sintra, Portugal. Magnesium sulfate (anhydrous technical grade) was obtained from Appli Chem GmbH, Darmstadt, Germany. Whatman® filter papers were obtained from Whatman International Ltd., England. All the chemicals and enzymes were used directly as received from the supplier without any further modification.

2.2. Experimental setup

The experimental setup involved the use of water bath with mechanical stirring, rotary vacuum bath and ultrasonic bath. The water bath (WB) used in the work was obtained from Grant Instruments (Cambridge) Ltd., England (model OLS 200) equipped with an orbital shaker and temperature controller. An ultrasonic bath (US) (USC600TH, VWR International Ltd., USA) with frequency 45 kHz and power of 120 W was also provided. The rotary vacuum bath (Vac) (Hei-VAP Advantage, Heidolph Instruments GmbH & Co., Germany) equipped with water bath and temperature controller was also used in the reactions. During the experiments, both the water bath and the vacuum bath were operated at 100 rpm.

The reaction mixture consisted of equimolar ratio of diethyl glutarate (10 mmol, 1.90 g) and ethylene glycol diacetate (10 mmol, 1.40 g) for a total reaction volume of 3 mL without any addition of solvents.

In the conventional approach, the reaction mixture with biocatalyst was subjected to a desired temperature in a water bath set at 100 rpm and the reaction time was 18 h at 40 °C [3]. The ultrasound assisted approach was performed by incorporating ultrasonic bath instead of water bath with mechanical stirring to establish the impact of ultrasound on the polyesterification reactions. The required reaction time in the ultrasonic bath operating at frequency of 45 kHz, 120 W power rating and duty cycle of 75% (15 min on/5 min off) was observed to be 1 h. Temperature was controlled at 40 °C. The set of reactions are summarized on Fig. 1. Both reaction mixtures (containing the enzyme) were then subjected to vacuum in bath at 40 °C for 6 h with stirring at 100 rpm for the evaporation of byproduct ethyl acetate in order to favor the forward polyesterification reaction. For both the approaches, the effect of enzyme loading was investigated over the range of 1% (w/v) to 20% (w/v).

Controls of the polyesterification reaction were performed for both approaches without enzyme. To study the effect of vacuum on the polymerization reaction, a set of reactions in water bath with mechanical stirring and in ultrasonic bath were ended before the vacuum step.

For the improvement of the polymerization with different enzyme loadings, a 72 h reaction was performed in vacuum bath in order to check an increase of the reaction turn over. The reaction time was based on previous polymerization reactions performed by Liu et al. [3].

To study the effect of ultrasound during reaction improvement, 2 reactions in water bath were interrupted by 1 h of ultrasound every 24 h of reaction time. However both reactions were performed with ultrasound/vacuum bath cycles, the first cycle varied. One of the reactions was initiated with 1 h of ultrasound while the other with 24 h of vacuum bath. Reactions with 1 h of ultrasound prior to 71 h in vacuum bath and only 72 h in vacuum bath were performed as control of the effect of the ultrasound/vacuum bath cycles on the poly(ethylene glutarate) reaction.

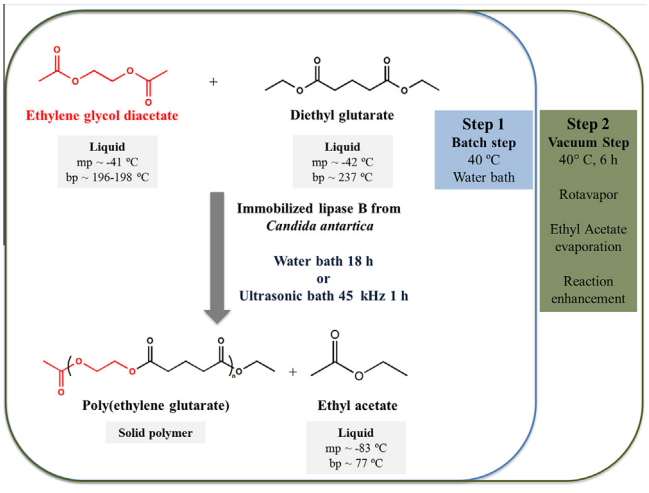

Fig. 1. Scheme of lipase-catalyzed solvent-free synthesis of poly (ethylene-glutarate).

2.3. Determination of Initial reaction kinetics

To determine the effect of ultrasound treatment on the initial reaction kinetics, 4 mL of the reaction mixture was incubated separately at 40 °C in the water bath and ultrasonic bath. To study the effect of the amount of enzyme on the initial kinetics, the reactions were performed using 1% and 10% (w/v) of enzyme loading. About 100 μL of reaction samples were taken out every 15 min and analyzed using GC-FID for the formation of ethyl acetate.

The concentrations of products were determined on a Chrompack CP-9001 Gas Chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector (FID). A CP-WAX 52 CB stainless steel column (25 m × 0.25 mm) was used for the analysis, packed with polyethylene glycol as stationary phase. Chloroform was used as mobile phase. Helium was used as carrier gas at a flow rate of 1 cm^3 min^{-1}. The temperature program used was as follows: initial temperature 60 °C for 0 min; 8 °C/min up to 250 °C and hold at 250 °C for 5 min. The injector and detector temperatures were kept at 300 °C. The concentration of ethyl acetate was determined for each withdrawn sample using a standard curve.

2.4. Purification of poly (ethylene glutarate)

To purify the obtained poly (ethylene glutarate), 3 mL of chloroform were added to the reaction mixture to dissolve the residual reagents and the synthesized polyester. After removing the immobilized enzyme by filtration, 15 mL of petroleum ether was added to precipitate the poly (ethylene glutarate). After evaporation of petroleum ether and chloroform, a mixture of tetrahydrofuran and petroleum ether in the proportion of 1:14 was added. The mixture was kept at −20 °C overnight to allow the synthesized polyester to precipitate. The mixture of polyester and residual reagents was then collected after evaporating the petroleum ether and tetrahydrofuran and samples were analyzed.

2.5. Determination of the degree of polymerization

The degree of polymerization of poly(ethylene glutarate) was determined using the ^{1}H NMR spectra on a 400 MHz Avance Bruker NMR Spectrometer (Bruker Co., Germany). The monomer conversion rate of polyesterification reaction was calculated using the Carothers' equation [21,26] as follows:

$$\overline{\mathrm{DP}n} = \frac{1}{1-\rho} \tag{1}$$

where; $\overline{\mathrm{DP}n}$ represents the degree of polymerization (DP) of polyester, and ρ represents the monomer conversion rate (MRC).

3. Results and discussion

The ultrasound-assisted solvent-free synthesis of poly (ethylene glutarate) from DG and EGD catalyzed by immobilized lipase was performed in the work. To compare the effect of ultrasound treatment on the enzymatic polyesterification of DG and EGD, the reaction was also performed in water bath with mechanical stirring at 100 rpm. The polyesterification reaction is represented in Fig. 1.

3.1. Effect of ultrasound on the initial reaction kinetics

To understand whether the sonication helps in the intensification of the reaction rate, initial reaction kinetics study was performed. The initial reaction kinetics were established for both the approaches and performed separately in a water bath with an orbital shaker and in an ultrasonic bath at 40 °C for 45 min, using Fermase CALB™ 10,000 as catalyst. Samples were periodically withdrawn, diluted in chloroform and analyzed using GC-FID. The production of ethyl acetate (EA) which is a side product of reaction was determined according the calibration correlation as given below (Fig. S1).

$$y = 160449.629 + 6.194E6x,\ R^2 = 0.984 \tag{2}$$

The initial reaction kinetics for the formation of EA is depicted in Fig. 2. The higher formation of product supports the enhancement in the initial rate in the presence of ultrasound. For both enzyme loading of 1% and 10% the formation of EA increased remarkably with the ultrasound treatment when compared with the conventional approach involving water bath with mechanical stirring. When the reactions were performed without enzyme there was no formation of EA.

The effect of the ultrasound treatment was more evident for the lower lipase concentration (Fig. 2). The observed intensification can be attributed to the elimination of mass transfer resistances due to the cavitational effects. The physical effects such as turbulence and acoustic streaming increases the probability of contact of the substrates with the catalyst and hence accelerates the rate of reaction [26,27]. For 1% (w/v) enzyme loading, the rate of the reaction performed in the water bath was almost zero as there was not a significant increase in EA concentration during the first 30 min of reaction. For the same enzyme loading, when the reaction was performed in the ultrasonic bath, the concentration of EA increased constantly along with time, clearly showing the intensification benefits of ultrasound treatment in the initial reaction kinetics. At the end of 45 min, the production of EA with the ultrasound treatment was around 6 times higher than that obtained with the water bath treatment (Fig. 2). While for 10%, it was around 1.8 times higher than that obtained using the water bath treatment (Fig. 2).

It was observed that the ultrasonic treatment favored the initial reactions when compared with the water bath treatment. It is reported that ultrasound has the influence to activate the enzymatic processes by providing the energy input which is not too harsh to disrupt the function of the enzyme and thus potentially favors the reaction [20,22].

The amount of enzyme also influenced the reaction kinetics. There was an increase of 1.63 folds in the formation of EA at the end of 45 min when the enzyme loading was increased from 1% to 10% (Fig. 2). As the enzyme loading increases, more active sites are available for the reaction which accelerates the reaction [26,28].

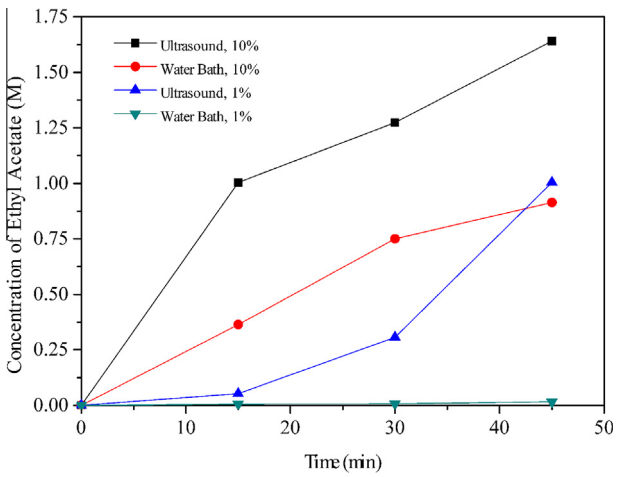

Fig. 2. Effect of ultrasound and water bath treatments in the production of ethyl acetate. Reactions were performed during 45 min at 40 °C and for two enzyme loadings (1% and 10% (w/v)).

3.2. Effect of ultrasound on degree of polymerization, monomer conversion rate and time of reaction

The degree of polymerization of poly (ethylene glutarate) was determined using the NMR spectra. A selected ^{1}H NMR spectra analysis is shown in Fig. 3. The degree of polymerization was determined based on the number of glutarate units from methylene absorptions at 1.911 or 2.347 ppm and number of ethylene from units from methylene absorption at 4.231 ppm. Low intensity signals attributable to $-OCH_2CH_3$ end group from methylene and methyl and $-C(O)CH_3$ end group from methyl were observed at 4.080 ppm, 1.213 ppm and 2.039 ppm, respectively.

To compare the ultrasound-assisted reaction with the conventional reaction process, reactions were performed in (the detailed approach has been described in the earlier section of materials and methods) water bath with mechanical stirring for 18 h or in the ultrasonic bath for 1 h, followed by 6 h in vacuum bath for each of the approaches. Longer reaction time in the ultrasonic bath did not improve the polymerization reaction (Table S1).

As shown in Fig. 4, for the same enzyme loading and the same temperature, 1 h of ultrasonic treatment resulted in similar effects as compared to the 18 h of water bath reaction giving similar degrees of polymerization (Fig. 4A) and monomer conversion rates (Fig. 4B) with the ultrasound treatment.

For enzyme loadings higher than 5% there was not an increase on the degree of polymerization (Fig. 4A) and on monomer conversion rates (Fig. 4B) as result of the exhaustion of the DG and EGD reagents and the single ethylene glutarate ester product. The exhaustion of the reagents and product changed the linear progression of the reaction as observed for enzyme loadings lower than 5%.

The mechanism of ultrasonic assisted intensification is based on the formation of micro-bubbles with vapors of reagents, which collapse due to the shock waves leading to a remarkable increase in both the local temperature and pressure coupled with physical effects. The alternate compression and rarefaction cycles and the cavitational effects also cause oscillatory fluid motion called micro-streaming that leads to extreme micro-mixing in the reaction medium. The overall effect gives the enhanced reactivity of reagents present in the system increasing the product formation in a short time [26].

No products were detected when the reaction was performed in the absence of immobilized enzyme. When the polyesterification of DG and EGD was done without vacuum the reaction was incomplete for the lower enzyme loadings. For the higher enzyme loadings, however no reagents were detected at the end of reaction, the maximum degree of polymerization was one. The presence of reagents at the end of reaction and the low degree of polymerization when the reaction was performed without the vacuum step confirm the effect of vacuum in favor the forward polyesterification reaction.

The ultrasound-assisted synthesis of polyester reduced the reaction time to 7 h for all enzyme loadings. The observed significant reduction in the reaction time for the polymer synthesis supports the application of ultrasound for the intensification of enzymatic polyesterification. Additionally, ultrasound can also increase the polyester production and give better monomer conversion rate compared to the traditional method of enzymatic polyesterification.

3.3. Improvement of the poly (ethylene glutarate) polymerization reaction

A series of long-term reactions in vacuum bath, interrupted by 1 h of ultrasound treatment during the reaction processing, were performed with different enzyme loadings at 40 °C for 72 h. The results depicted in Fig. 5 indicate that 1 h of ultrasound treatment at the very beginning of the long-term reaction processing is helpful for enzyme loading below 15% (w/v). On the contrary, a lower degree of polymerization was obtained for the highest degree of enzyme loading (20% (w/v)) for the reactions irradiated with ultrasound at the beginning and during the reaction process. The degree

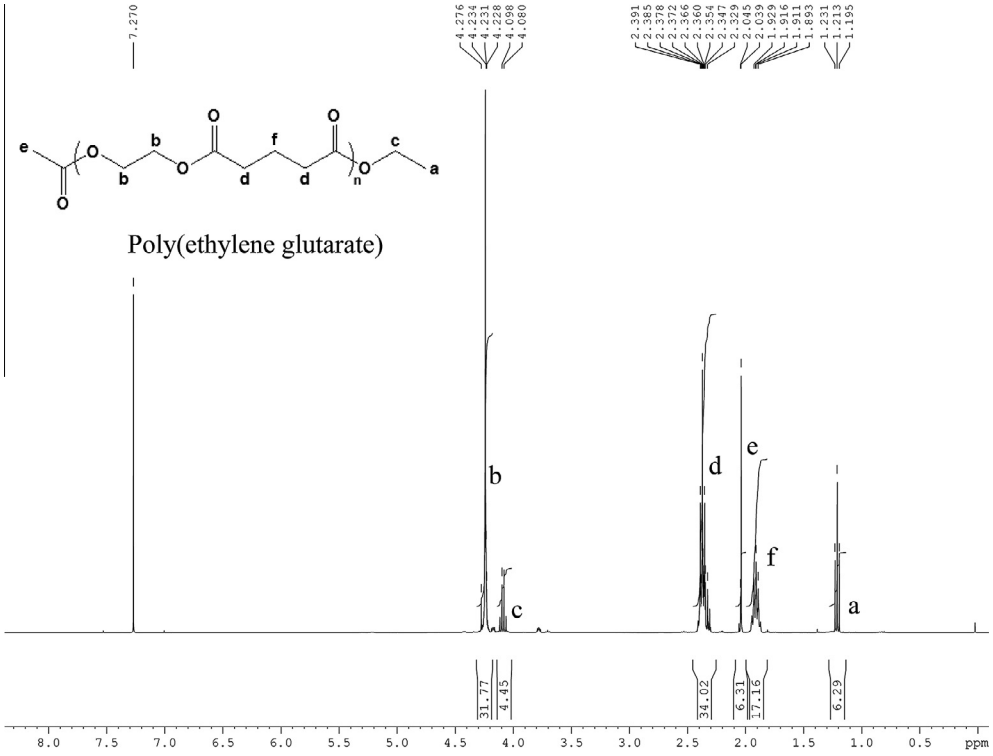

Fig. 3. ^{1}H NMR spectra of poly(ethylene glutarate) in $CDCl_3$, Reaction was performed in the water bath for 18 h at 4040 °C, and then transferred to the vacuum bath for 6 h at 4040 °C with an enzyme loading of 7% (w/v).

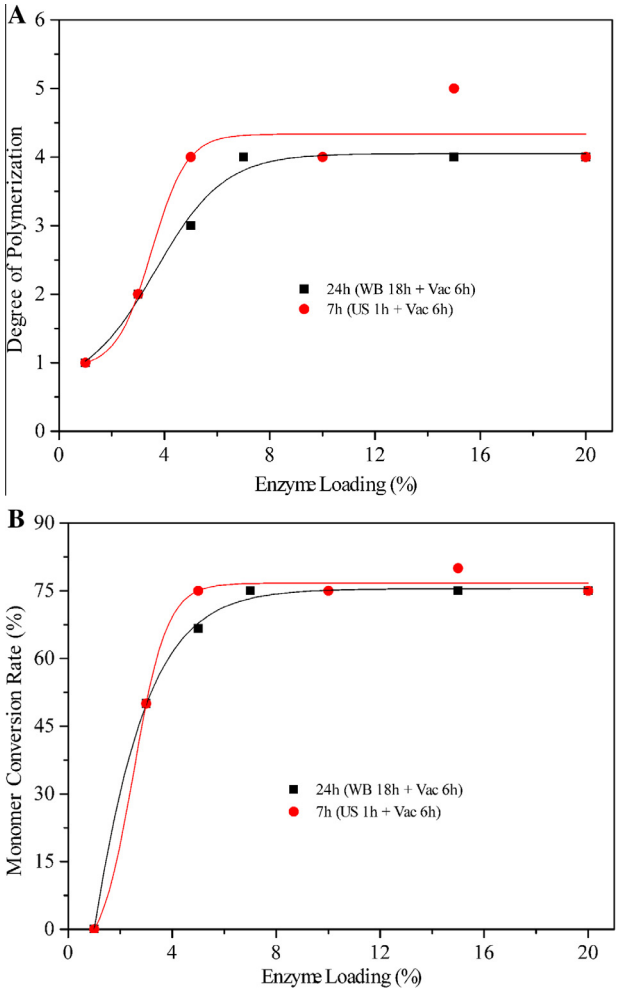

Fig. 4. Effect of reaction treatments on degree of polymerization (A) and monomer conversion rate (B). Reaction were performed at 4040 °C separately in the water bath for 18 h and in the ultrasonic bath for 1 h followed by 6 h of operation in the vacuum bath.

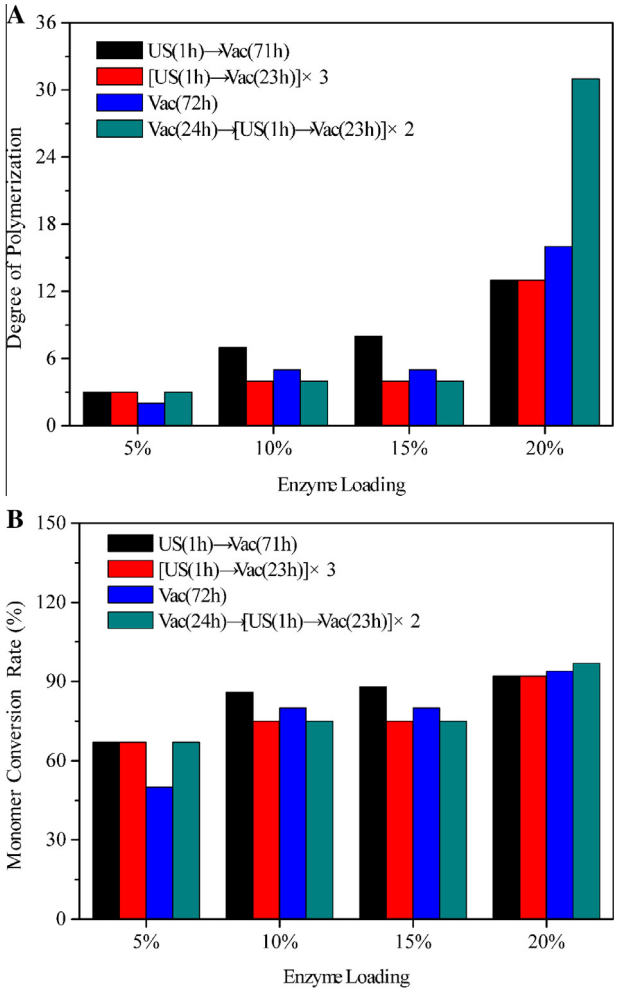

Fig. 5. Optimization of reaction process for the production of poly(ethylene glutarate). The effect of long term reaction and type of treatment was optimized regarding the degree of polymerization (A) and monomer conversion rate (B). Reactions were performed at 40 °C for 72 h.

of polymerization was significantly improved for reaction batch with 20% (w/v) enzyme loading when sonicated twice for 1 h during 72 h of continuous process and not only irradiated at the very beginning of reaction. The highest degree of polymerization as 31 and a monomer conversion rate of 96.77% were achieved with 20% enzyme loading at the end of 72 h at 40 °C. It has been also observed that the rate of polymerization gradually declines with time. The apparent reason for the observed trend can be that as the polymer concentration and average weight of polymer in the reaction mixture increases, the viscosity of the reaction mixture also increases which results in reduced cavitational activity and hence the degree of intensification. Secondly, as the size of the polymer molecules build up with time, the diffusive transport of the polymer molecule reduces (irrespective of the viscosity of the solvent) giving slower reaction rate [25]. The ultrasound treatments during the reaction process temporarily disaggregate the enzyme bulk formed due to the high enzyme loading improving the amount of enzyme available for the reaction [26,29].

The reaction might be further improved if a combined vacuum/ultrasound set up would have been used. Those experiments were not performed for security reasons and completely new set-up would be need to be build, rather than just leave the ultrasonic bath under rotavapor apparatus. This would be subject of a future study by our group.

4. Conclusions

Using an ultrasound-assisted lipase-catalyzed process, polyester poly (ethylene glutarate) was synthesized in a solvent-free condition. The reaction kinetics for the production of ethyl acetate established the notable intensification of the ultrasound on the condensation polymerization. The reaction time was reduced from 24 h to 7 h for lower enzyme loadings when the conventional water bath treatment was replaced with the ultrasound treatment followed by 6 h in the vacuum bath. The reduction in the reaction time established the positive effect of ultrasonication on the poly (ethylene glutarate) reaction meeting the demand for green chemistry and a sustainable development. The highest degree of poly (ethylene glutarate) polymerization and monomer conversion rate, respectively 31 and 96.77%, were obtained for a long-term reaction with 1 h of ultrasound treatment for every 24 h of reaction in the water bath and enzyme loading of 20% (w/v).

The present work shows the potential of ultrasound and vacuum treatment in solvent-free enzymatic synthesis of poly (ethylene glutarate) from ethylene glycol diacetate and diethyl glutarate. The ultrasound treatment reduced the time of reaction compared with the traditional methods, and gave higher degree of polymerization and better monomer conversion rate in a shorter period.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ultsonch.2016.02.005.

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